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14. ABSTRACT
The androgen signaling pathway remains a key therapeutic target for prostate cancer. Despite recent advances in treatment, disease invariably recurs. Genes regulated by androgen receptor (AR) in recurrent prostate cancer differ from those in normal cells or early disease. A novel goal is to inhibit AR target genes involved in cancer growth but retain expression of genes for normal cell survival. These gene sets may differ in androgen response element (ARE) signatures. AREs include consensus inverted repeats (cARE) shared amongst receptors and more selective direct repeats (sAREs). Genes involved in differentiation appear to rely more on sAREs than genes driving proliferation. ARE preference can vary with ligand, cofactor interactions or AR mutation. Thus compounds altering receptor conformation via any domain may influence promoter choice. To identify such compounds, we developed a multiplexed assay for differential AR action in transfected cells using fluorescent reporters driven by multimerized cAREs, sAREs or a PSA promoter. We performed a high-throughput screen of a library of FDA-approved drugs, seeking candidates that suppressed cARE but not sARE reporters, and are currently narrowing hit compounds for validation. This promoter-dependent screen may identify compounds that elicit differential gene expression and lead to drugs generating fewer side effects and less resistance.

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MULTIPLEXED PROMOTER-DEPENDENT SCREEN FOR SELECTIVE ANDROGEN RECEPTOR MODULATORS

INTRODUCTION

Androgens work via the androgen receptor (AR) to play a key role in neoplastic as well as normal prostate growth and therefore the androgen signaling pathway remains a high impact therapeutic target. Antiandrogen therapy is initially successful but tumors ultimately progress to hormone resistance. In these castration-recurrent tumors, AR levels remain high and AR signaling persists, indicating that disease remains dependent on AR (1). Recent progress towards more complete blockade of androgen synthesis (i.e., by abiraterone) and more robust AR antagonism (i.e., by MDV3100) should improve clinical outcomes but resistance may still arise from ligand-independent or variant ARs (2, 3). The goal of our project was to identify AR antagonists with differential actions (selective AR modulators – SARMs) that would inhibit expression of genes involved in cancer cell growth but retain expression of genes involved in cell survival and differentiation. As confirmed by recent genomic analyses, genes regulated by AR in normal cells or in early stage disease differ from those targeted in castration-recurrent prostate cancer (4). Our underlying hypothesis is that these different sets of genes are coordinately regulated due to promoters that share certain androgen response elements (AREs) and chromatin signatures. AR response elements can be generally characterized as those that are consensus elements shared with other steroid receptors (cAREs; exemplified by the inverted repeat HRE3) or those that are more selectively recognized by AR (sAREs; exemplified by the direct repeat HRE2). Several lines of evidence suggest that the sAREs may be more critical for genes involved in differentiation (5, 6). AR binding to different response elements is affected by DNA sequence, cofactor interactions and ligand structure, all of which impact receptor conformation. Thus compounds interacting with any receptor domain could influence choice of target gene. We proposed a high-throughput multiplexed promoter-dependent screen to identify SARMs that elicit differential gene expression. A similar approach previously identified differential modulators of the glucocorticoid receptor (7). Resulting compounds have promise in reducing resistance as well as producing fewer side effects in treatment.

BODY

The grant outlined 3 tasks in the Statement of Work. Developing and optimizing the screen took longer than expected due to difficulties with instrumentation, but with some modifications the first screen of a library of FDA-approved compounds has been performed. We intend to rescreen possible hits, validate them individually and follow up on interesting compounds. Relevant details of the development of the screening assay are described below.

Task 1. Construct and test fluorescent reporters with distinct promoters.

Construct Reporters. The multiplexed promoter screen utilizes 4 promoters: multimerized cAREs driving a minimal thymidine kinase (tk) promoter, multimerized sAREs driving tk, a PSA enhancer/promoter (containing both cAREs and sAREs, and binding sites for other transcription factors), and the SV40 promoter (as a control lacking hormonal response). These promoters were cloned upstream of fluorescent protein (FP) reporter genes for mCherry, citrine, mOrange2 or cerulean, obtained from Roger Tsien (UCSD) or Joel Swanson (UMichigan). This

was accomplished by excising luciferase from pGL3-basic ARE reporters by digesting with NcoI and FseI, and replacing with FPs that had been amplified by PCR with primers containing NcoI or FseI restriction sites. For flexibility in determining the optimal ARE-FP combinations for screening, each FP was cloned downstream of each hormone-responsive promoter (cARE, sARE, PSA), resulting in 12 unique reporters (see Fig. 1A, B). To control for off-target and nonspecific effects, LacZ in the SV40-βgal vector was replaced with mCherry. Vector accuracy was verified by DNA sequencing.

Host Cells and Test Transfections. We had originally proposed screening with VCaP cells, a well-characterized prostate carcinoma cell line with amplified AR, but these cells proved difficult to transfect and did not withstand the manipulations required for high-throughput screening. As an alternative, two HeLa cell lines expressing different levels of stably transfected AR were obtained from Dr. Elizabeth Wilson (8). AR expression in the 'high" AR cell line (HeLa-AR) is similar to VCaP (Fig. 1C) and was chosen for screening. Importantly these cells contain an integrated PSA-luciferase reporter gene, eliminating the need to transfect a fluorescent PSA reporter. HeLa-AR cells transfect well and are sufficiently robust for screening. Subsequent validation of hit compounds will be done in prostate carcinoma cell lines.

Using a fluorimeter with filters that read eGFP, mOrange and mCherry, several of the FP vectors were tested for responsiveness to hormone following transfection into HeLa-AR cells. Cells were plated in charcoal-stripped serum and treated with 100 nM R1881. As shown in Fig. 1D, cARE and sARE responded to hormone while SV40-driven mCherry levels were unaffected.

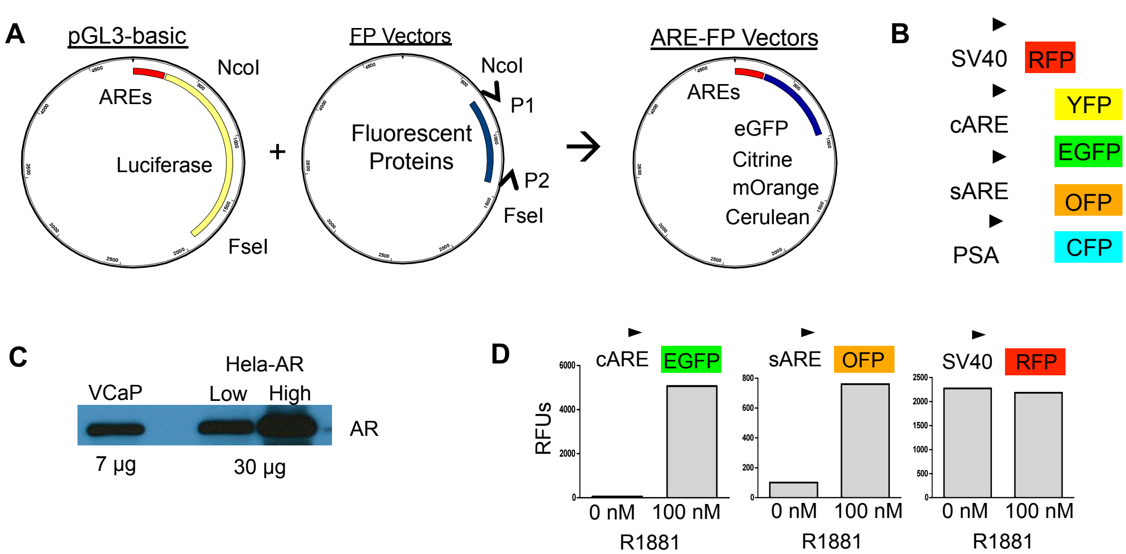

Fig 1. Reporters and host cells for screening. **A)** The androgen response elements (sAREs, cAREs, PSA) are upstream of luciferase in pGL3-basic vectors. Luc was removed using NcoI and FseI restriction sites (*left*). Each fluorescent protein (FP) coding region was amplified by PCR with primers (P1, P2) containing NcoI and FseI sites (*middle*). Each FP was inserted downstream of the AREs in pGL3 (*right*), generating 12 unique vectors (YFP – Citrine, OFP – mOrange, CFP – Cerulean) (**B**). A constitutive SV40-mCherry reporter (SV40-RFP) was generated similarly. **C)** Relative AR expression in VCaP and two HeLa lines with stably transfected AR. Whole cell lysates were resolved by SDS-PAGE and membranes from transferred gels probed with anti-AR (AR-N20) and rabbit secondary antibody. Note amounts of lysate loaded differ. **D)** HeLa-AR cells (high) were transiently transfected with cARE-eGFP, sARE-OFP or SV40-RFP, and treated with 100 nM R1881 or no hormone for 24 hrs. Lysates were read at appropriate excitation/emission wavelengths on a flourimeter. Data is in relative fluorescent units.

Task 2. Perform high-throughput promoter-dependent screen of library of compounds.

Screen Development. The fluorescent protein reporter assay was translated from 12-well to 384-well high-throughput screening format (HTS) at the University of Michigan Center for Chemical Genomics (CCG). Mass handling of cells was optimized for transfection efficiency and induction ratio, and several different fluorescent plate readers were tested for most sensitive signal detection and least overlap between wavelengths. These parameters were tested first for single reporters and subsequently for simultaneously transfected reporters in order to develop a multiplex assay.

The following protocol was used for all HTS experiments:
Day 1 – plate HeLA-AR cells in 10 cm dishes to adhere overnight.
Day 2 – transfect cells with reporters, alone or in combination, with xTREMEGENE HP transfection reagent.
Day 3 – replate cells into 384-well plates with a multidrop dispenser; treat with 100 nM R1881, 100 nM R1881 plus 100 μM bicalutamide, or no additional treatment, for 24 hrs.
Day 4 – read fluorescent reporter protein expression or luminescence (for endogenous PSA-luc).

Likely due to all the additional manipulations, HeLa-AR cells fared poorly in charcoal-stripped serum, which was used initially to maximize hormone induction over basal gene expression. Since compounds are screened in the presence of saturating levels of ligand, assays could be performed in the presence of complete serum (FBS) instead. Some reporters showed further increased expression with 100 nM R1881, and all were suppressed by bicalutamide. Bicalutamide suppression was used to indicate reporter function.

The CCG has several instruments for reading fluorescent assays. We originally intended to use the EnVision multilabel plate reader that allows rapid assay but the ARE-FP reporters failed to produce sufficient fluorescence to be detected above background. In contrast, the ImageXpress Micro Cellular Imaging and Analysis System (IXM) allowed sensitive and quantitative fluorescent image detection using specialized software. As shown in Fig. 2, FPs driven by AREs are increased in expression by R1881 and suppressed in a dose-dependent manner by antagonist, whereas mCherry driven by SV40 is insensitive to hormone. Unfortunately, when multiple reporters were cotransfected, signals were diminished and there was considerable crosstalk in fluorescence amongst the reporters, precluding use of the IXM for multiplex screening.

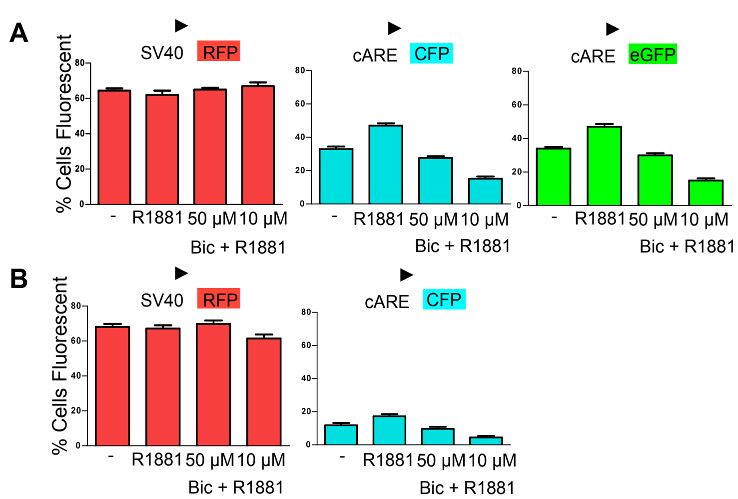

Fig 2. ARE-FP activity as read by ImageXpress Analysis System. **A**) HeLa-AR cells were transfected with SV40-mCherry (RFP), cARE-cerulean (CFP) or cARE-eGFP, re-plated into 384-well plates and treated with R1881 (100 μM) in the absence or presence of bicalutamide (Bic; 50 or 100 μM). Cells were fixed after 24 hrs, stained with DAPI and imaged using the IXM. Data were analyzed using MetaXpress software. **B)** Same as in A, except HeLa-AR cells were co-transfected with SV40-mCherry and cARE-CFP (5 mg each). Data is represented as percent of cells that are fluorescent.

We next tested the ability of the EnSpire multi-mode monochromator-based plate reader to distinguish different fluorescent reporters compared to the filter-based EnVision reader. As shown in Fig. 3A, both cAREs and sAREs upstream of citrine (YFP) were strongly induced in response to agonist, an effect that was completely antagonized by bicalutamide. EGFP read more poorly, with less activation and suppression compared to basal expression in FBS, and cerulean and mOrange failed to read above baseline. For the endogenous PSA-luciferase in the HeLa-AR cells, high baseline luminescence due to plating in FBS was not induced further by R1881 but there was complete suppression by bicalutamide, readily detected on the PHERAstar microplate reader, allowing use of PSA-luc in a screening assay. The PHERAstar can also be fitted with a DsRed filter to assess mCherry activity. This would allow multiplex reading of FPs on the EnSpire and subsequent reading of SV40-RFP and PSA-luc on the PHERAstar.

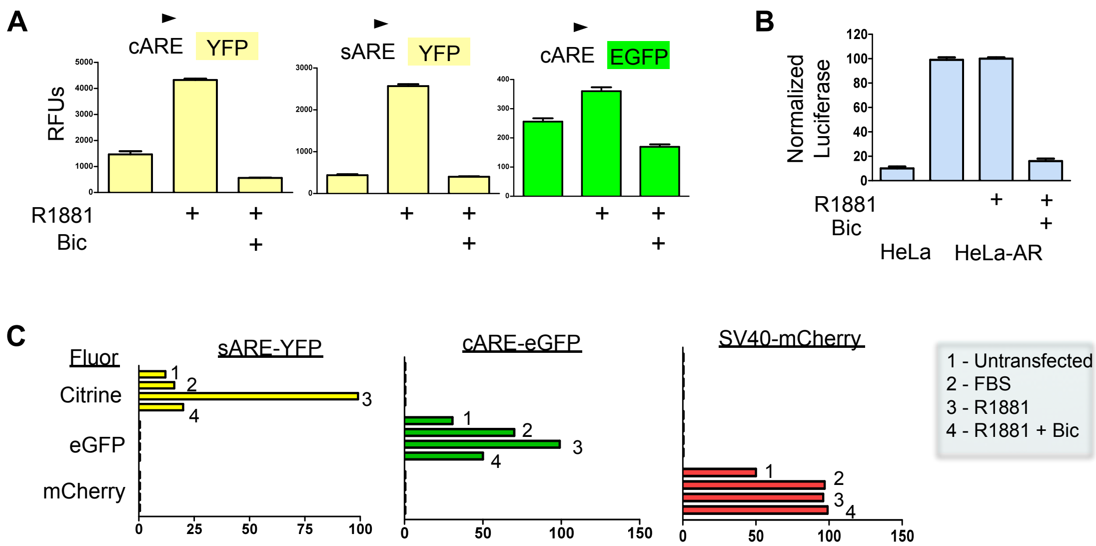

Fig. 3. Activation of single reporters in HeLa-AR cells. **A)** HeLa-AR cells were transfected with cARE-citrine (YFP), sARE-citrine or cARE-eGFP and treated with R1881 (100 nM) in the absence or presence of bicalutamide (Bic, 100 μM) for 24 hrs in 384-well plates. Fluorescence was detected on an EnSpire monochromator. Values are in relative fluorescent units (RFUs). **B)** PSA-luciferase was assayed in wild-type HeLa (*left bar*) or HeLa-AR cells plated in 384-well plates and treated with R1881 (100 nM) in the absence or presence of bicalutamide (100 μM) for 24 hrs. Cells were lysed in Steady-Glo luciferase reagent, luminescence read on the PHERAstar and normalized to level of R1881-induced activity. **C)** Absence of crosstalk between ARE-FP reporters. HeLa-AR cells were transfected with sARE-YFP, cARE-eGFP or SV40-mCherry and treated with R1881 (100 nM) in the absence or presence of bicalutamide (100 μM) in 384-well plates. Each well was read at the optimal settings for each FP. Untransfected cells served as background controls. Data are represented as normalized fluorescence relative to R1881.

To determine crosstalk in reading multiple fluorescent reporters, sARE-citrine, cARE-eGFP and SV40-mCherry were individually transfected into HeLa-AR cells and fluorescence at each setting was read sequentially on the EnSpire (YFP, eGFP) and PHERAstar (mCherry) plate readers (Fig. 3C). Reporter fluorescence was not detected at nonoptimal settings (i.e., eGFP and mCherry do not read in the citrine channel), confirming absence of crosstalk among these FPs.

To determine strength and specificity of signals when reporters are multiplexed, HeLa-AR cells were transfected with sARE-YFP (4 μg), cARE-eGFP (4 μg) and SV40-mCherry (2 μg). Following treatment, fluorescence was analyzed at each FP setting using the EnSpire and

PHERAstar readers. Although sARE-YFP and cARE-eGFP were activated by hormone and repressed by bicalutamide (Fig. 4), the magnitude of the difference, most notable for citrine (YFP), was greatly diminished compared to reporters transfected individually (see Fig. 3). Z scores, calculated for each reporter to define assay quality, suggest this strategy would not sufficiently distinguish the effect of a compound from background noise (Z' < 0.5).

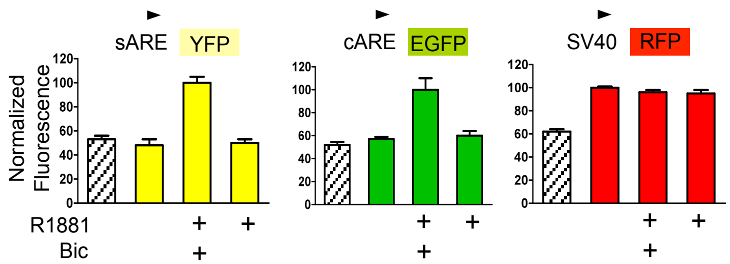

Fig. 4. Multiplexed ARE-FP activity. HeLa-AR cells were co-transfected with sARE-YFP (4 µg), cARE-EGFP (4 µg) and SV40-mCherry (2 µg). Fluorescence was analyzed following 24 hr treatment with R1881 (100 nM) in the absence or presence of bicalutamide (Bic; 100 µM). Hatched bars represent background of untransfected cells. Fluorescence is normalized to R1881-induced levels.

Pilot Screen. To achieve the best separation between positive (R1881) and negative (bicalutamide) controls, thus increasing ability to identify hit compounds, we chose to screen cARE and sARE separately, each multiplexed with PSA-luciferase. Citrine was the optimal FP for each ARE, showing the greatest activation by agonist and repression by antagonist (Fig. 3A). We first ran a pilot screen to ensure that the signal window between controls was maintained in the presence of 0.5% DMSO, the vehicle used for the compound library. Cells transfected with cARE- or sARE-YFP were treated with R1881 and 0.5% DMSO and every other column in the 384-well plate received bicalutamide. Each reporter was 4- to 6-fold repressed in response to bicalutamide (Fig. 5). Z' scores were 0.66 for cARE-citrine, 0.53 for sARE-citrine and 0.62 for PSA-luciferase, acceptable characteristics for HTS.

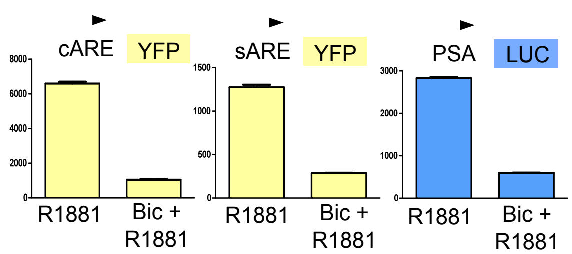

Fig. 5. Pilot screen shows suitability of reporters for high-throughput assay. HeLa-AR cells were transfected with sARE-Citrine (YFP) or cARE-Citrine (YFP) and treated with R1881 (100 nm) in the absence or presence of bicalutamide (100 µM) in 384-well plates. Fluorescence was read on the EnSpire. Luciferase was activated by addition of Steady-Glo reagents and luminescence was read on the PHERAstar.

We performed a HTS screen of the Spectrum FDA-approved compound library, a collection of over 2000 small molecules, and the NIH library of 450 compounds that have been used in clinical trials. About half of the compounds in the Spectrum library have been used in human therapy, another third are natural products and derivatives with undetermined biological activities, about 15% have had experimental activities reported, and 70 are approved and restricted to agricultural use. Each 384-well plate in the assay contained 2 columns of positive control (suppression by bicalutamide) and 2 columns of negative control (stimulation by R1881); each well of the columns in between received a test compound at 25 µM concentration.

Of the ~2500 compounds, fewer than 1/10th caused greater than 75% suppression of AR-driven promoter activity, a number expected to include false positives, such as compounds that were generally toxic or non-specific in effect. Known compounds within the libraries serve as positive controls. For example, the test bicalutamide suppressed all ARE reporters and was most potent against sAREs. Test flutamide was less efficacious and was most potent on the PSA reporter. We used 75% suppression of ARE-driven citrine fluorescence, and a level of less than 300 counts of PSA-luciferase, as values to begin to identify AR antagonists. As shown in Fig. 6, of about 200 compounds that suppressed AREs more than 75%, about 3/4 suppressed both cAREs and sAREs to similar extents. When PSA suppression is included in analysis, more evidence for differential effects of compounds appears. To ask more stringently whether some compounds suppress selectively, we tallied compounds that suppressed one ARE by more than 75% but the other by less than 25%. Interestingly, 22 compounds appeared to strongly suppress cAREs but have little activity against sAREs – these are the compounds we are most interested in as potentially able to suppress cell proliferation but not differentiation. Furthermore, 8 compounds had the opposite phenotype and suppressed sAREs but not cAREs. While this is a first pass and requires validation prior to further characterization of compounds, the results are of a magnitude in line with our expectations, particularly in finding compounds that selectively inhibit cAREs but not sAREs, and a smaller set with the opposite preference.

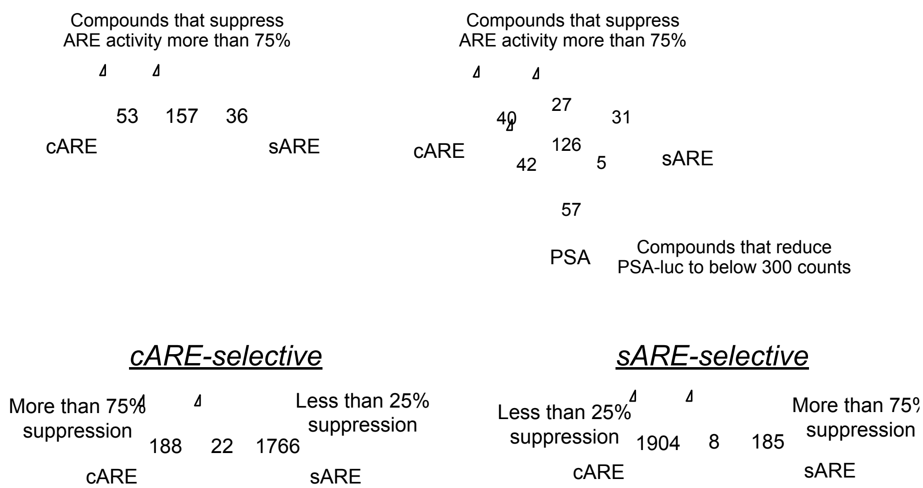

Fig. 6. Venn diagrams showing overlap in sets of compounds (by number) that strongly suppress both consensus and selective AREs (top left), AREs and the PSA promoter (top right), or compounds that more stringently suppress one type of ARE (e.g., cARE) but have little effect on the other (e.g., sARE). cARE-selective compounds are of most interest for this project.

As an indication of the validity of this screen, among the 8 compounds that suppress sAREs more effectively than cAREs are cyproterone acetate and medroxyprogesterone acetate, the first of which was clinically used as an antiandrogen in the past and the second is a progestin that inhibits AR at high doses. Among the compounds of greater interest that inhibit cAREs but not sAREs are several flavonoids and anthracyclines, some of which are known to inhibit AR. Promising compounds will be re-screened, and dose response and specificity determined.

Task 3. Validate effects of compounds on gene expression profiles.

This task will be completed once promising hit compounds to pursue are selected. There are external as well as institutional sources identified for potential additional funding.

KEY RESEARCH ACCOMPLISHMENTS

- We have created a series of reporters to assess promoter-selective effects of AR agonists and antagonists.
- We have developed a compound high-throughput screen that uses multiplexed and sequential reporter assays to identify selective AR antagonists.
- We have completed a first HTS assay of 2000 FDA-approved drugs and 450 NIH compounds.
- We are analyzing the primary screening data to identify a small set of promising leads for further development.
- The screen is capable of identifying Selective AR Modulators that have differential effects dependent on promoter.

REPORTABLE OUTCOMES

I presented a Plenary address at the Endocrine Society Symposium that described previous work from the lab upon which this proposal was based:

06/06/2011 – Boston, MA - Genetic Variation of the Androgen Receptor: from Gene Regulation to Prostate Cancer

Compound information will be made publicly available once reported via publication.

CONCLUSION

Novel AR antagonists, or SARMs, have promise in inhibiting oncogenic functions of AR but sparing activities promoting normal cell growth and survival. Based on the premise that selective AR response elements are more frequently associated with genes involved in differentiation than proliferation, we developed a promoter-dependent screen for AR antagonists with greater activity on consensus than selective ARE-driven reporter genes. Optimization of the screen took significant effort, with best results obtained for sequential screening of consensus versus selective ARE-driven reporters, each multiplexed with a PSA-luciferase reporter. Screening ~2500 compounds, each with some characterization by the FDA or NIH, resulted in an initial pool of about 200 compounds that showed reporter suppression. Of these, about 20 preferentially suppress cAREs and 8 suppress sAREs. These and other compounds from the screen will be further studied. The ultimate goal is to derive an AR antagonist with greater tumor-specific activity but less prone to development of treatment-limiting resistance or side effects.

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